The impact of kosmotropes and chaotropes on bulk and hydration shell water dynamics in a model peptide solution

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Abstract

Kosmotropic (order-making) and chaotropic (order-breaking) co-solvents influence stability and biochemical equilibrium in aqueous solutions of proteins, acting indirectly through the structure and dynamics of the hydration water that surrounds the protein molecules. We have investigated the influence of kosmotropic and chaotropic co-solvents on the hydrogen bonding network dynamics of both bulk water and hydration water. To this end the evolution of bulk water and hydration water dynamics of a prototypical hydrophobic amino acid with polar backbone, N-acetyl-leucine-methylamide (NALMA), has been studied by quasielastic neutron scattering as a function of solvent composition. The results show that bulk water and hydration water dynamics, apart from a dynamical suppression that depends on the NALMA solute, exhibit the same dependence on addition of co-solvent for all of the co-solvents studied (urea, glycerol, MgSO₄, and dimethyl sulfoxide). The hydrophobic solute and the high concentration water-structuring additive have the same effect on the water hydrogen bonding network. Water remains the preferential hydration of the hydrophobic side chain and backbone. We also find that the reorganization of the bulk water hydrogen bond network, upon addition of kosmotrope and chaotrope additives, is not dynamically perturbed, and that the hydrogen bond lifetime is maintained at 1 ps as in pure bulk water. On the other hand the addition of NALMA to the water/co-solvent binary system causes reorganization of the hydrogen bonds, resulting in an increased hydrogen bond lifetime. Furthermore, the solute's side chain dynamics is not affected by high concentrations of co-solvent. We shall discuss the hydration dynamics results in the context of protein folding and protein–solvent interactions.
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1. Introduction

Protein unfolding occurs when the balance of forces between the protein–protein interactions and the protein–solvent interaction is disrupted. The disruption may be the result of a perturbation of water structure and dynamics around the protein. Water molecules in the vicinity of a biomolecule may be classified in three categories: internal water, hydration water and free water. Internal water, or structural water, is a relatively immobile water within the solute, and its dynamics is often restricted to slow rotation [1–3]. Hydration water consists of structured water shells that depend on the protein surface interaction; its diffusion dynamics is slow, and it can exchange with outer shells [4–11]. Free water is water of and beyond the third hydration shell, which in principle is not strongly influenced by the protein surface other than due to the excluded volume effect of the protein molecule [4]. In principle the internal and hydration water are more important for protein stability and function. Water structure and dynamics can be perturbed either through temperature or pressure, or through kosmotropic (order-making) and/or chaotropic (order-breaking) co-solvents [12]. Kosmotropic co-solvents are very soluble compounds that are well hydrated with strong hydrogen bonds to water molecules. Kosmotropic substances decreases the solubility of hydrophobic particles and stabilize their aggregates and are excluded from the

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immediate surrounding of non-polar solute particle. Chaotropes are poorly hydrated co-solvents that break down hydrogen bonds and are thought to interact directly with protein. They increase the solubility of hydrophobic particles and encourage protein extension and denaturation [13–15]. Several hypotheses have been offered to explain the effects of solutes on protein stability. The principal hypothesis states that stabilisers and destabilisers of globular protein act indirectly by altering water structure and dynamics, but other hypotheses suggest that this is not the determining factor and that other effects such as excluded volume, affinity for protein surface, and ability to attenuate or accentuate the hydrophobic effect, should be considered [16].

In this paper we address the question of how the hydrogen bonding network dynamics of *bulk* and *hydration water,* together with protein side chain dynamics, may be influenced at the molecular level by water structure stabilizing (kosmotropic) and destabilizing (chaotropic) co-solvents. Considering the complexity of the whole system and experimental limitations for studying molecular events in the dynamics of a protein's first hydration shell we use a simplified model system.

Experimental limitations arise from several factors. Water–monomer interactions fluctuate on a short time scale and involve many complex factors such as hydrogen bonds and hydrophobic interactions, and different types of water confinement. One of the principal limitations of these studies is that the most important techniques to investigate the water dynamics give information averaged over all kinds of interactions. Thus it is impossible to distinguish between contributions from hydrophobic and hydrophilic sites or between regions that are more or less exposed to the solvent, and therefore difficult to characterize with confidence events at the molecular level [17]. Another real limitation has to do with the molecular environment. Highly concentrated solutions, adapted to have only one or two hydration water layers around the biomolecule, are difficult to obtain *in vitro* due to aggregation and precipitation. On the other hand dilute solutions are difficult to study because contributions from bulk water dominate and overwhelm the interesting effects due to hydration water.

In our recent work the approach has been to separate out different types of water dynamics, involving hydrophilic/hydrophobic interactions, and backbone/local side chain interfaces, through the study of model peptide systems whose dimensions are of the order of several water diameters. Short simple peptides are highly mobile, and the central side chain is forced to interact with solvating water. In addition, the possibility of end-capping the short sequence helps to reduce the charge effect and the choice of simple blocks is important in allowing the side chain to visit many possible solvation states. The side chain's effects on the structure of water become more pronounced as the surface of the residue accessible to the solvent increases [18]. Infrared spectroscopy studies show that the size of water clusters around the side chains of hydrophobic

amino acids increases in the following order: Gly < Ala < Val < Ile, Leu [19]. Russo et al. have controlled protein surface inhomogeneity by characterizing the dynamics of the first interacting water molecule near a completely homogeneous hydrophobic oligo-peptide, penta-Alanine, which adopts a beta-sheet conformation [1]. Then, in order to study the hydrophobic effect on the first hydration layer in solution, we considered the dynamics of hydration water near N-acetyl-leucine-methylamide (NALMA), a hydrophobic amino acid side chain attached to a blocked polypeptide backbone (equivalent to a Gly–Leu–Gly peptide, Fig. 1). Previous work, using X-ray scattering experiments and molecular dynamics simulations [20], on the structural organization of these peptides in solution, through the full concentration range from 0.5 M (1 mole NALMA to 110 moles H_2O) to 2.0 M (1 mole NALMA to 27 moles H_2O), revealed that water stabilizes either mono-dispersed or small clusters of amino acids, rather than causing complete segregation of the hydrophobic solute molecules into one large cluster. (Note that a 1.0 M solution has a concentration of 1 mol/L.) In addition small angle scattering experiments performed on NALMA in water over the same range of concentrations exclude the formation of NALMA aggregates [4].

Given this structural hypothesis a number of quasielastic neutron scattering experiments have been performed in order to study hydrophobic effects on water dynamics and solute dynamical relaxation as a function of hydration layer [21,22]. Because of its high solubility the NALMA–water system is ideally suited to studies of the dynamics of different hydration layers near a hydrophobic amino acid. High solute concentrations, such as 2M, simulate a biological interface with a shared water layer whereas more dilute concentrations, such as 0.5 M, have in principle enough water for 2–3 complete hydration layers surrounding each NALMA molecule. This solute configuration permits the separation of inner and outer hydration shells around a purely hydrophobic amino acid hydration site, enabling characterization of the first hydration layer dynamics and its influence on the outer layer dynamics [4,20]. Another interesting aspect of the high concentration solute with the corresponding single hydration layer is that it simulates a protein core, permitting investigation of the hydrophobic core dynamics, or the dynamics of the trapped water. These simple models are also important to study the influence of water molecule translational diffusion on protein dynamics and the role of protein–water
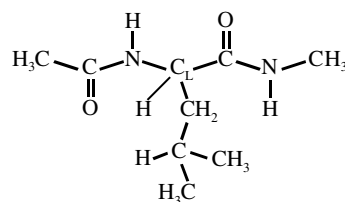

Fig. 1. The NALMA molecule.

hydrogen bonds [23,24]. The hydration layers of NALMA at room temperature exhibit diffusional dynamics corresponding to supercooled water at 260 K for the first layer and at 280 K when three layers are in place. This model therefore simulates a system where the bio-interface and the water are at two different temperatures. Thus to investigate the role of water molecule translations in protein structural relaxation the temperature of the water can be altered, either by changing the number of water layers or by adding co-solvents. Using molecular dynamics simulations with restraints applied to water molecule positions (which is equivalent to reducing the solvent temperature), Tarek and Tobias have studied the role of protein water hydrogen bond dynamics in the protein dynamical transition, and have shown that inhibiting solvent displacement has an effect on the protein that is similar to dehydration [24].

Our goal in the present study is to investigate and understand the influence of kosmotropic and chaotropic co-solvents on the hydrogen bonding network dynamics of *bulk* and *hydration water*. We report incoherent quasielastic neutron scattering measurements at room temperature of the dynamics of bulk water and hydration water in a 1.0 M solution of NALMA, perturbed by 2.5 M glycerol (a non-ionic kosmotrope), 5 M urea (chaotrope), 0.5 mM $MgSO_4$ (ionic kosmotrope) and 13 M dimethyl sulfoxide (DMSO, non-ionic kosmotrope). The urea–water system has the interesting property that it acts as a protein denaturant when used at very high concentrations such that urea molecules are readily available for hydrophobic solvation. In general, proteins start to unfold from their native structure at concentrations between 4.0 M and 5.0 M [25,26]. Glycerol and DMSO are instead used as cryoprotectants and have pharmaceutical and medical applications. Their effects on protein preservation have been the subject of several interesting studies [27–30]. High concentration of DMSO can depress water freezing point down to −70 °C while glycerol at 67% can get down to −46 °C. $MgSO_4$ is an ionic kosmotrope with different properties from a non-ionic kosmotrope, mainly due to the direct and polarized arrangement of surrounding water [31]. The 1 M NALMA offers an extended and well-structured water shell which permits an understanding of how kosmotrope and chaotrope additives interact with the hydrophobic hydration layer perturbing its dynamics.

We observe unexpected features in the dynamics of these biological solutions under ambient conditions. The results show that bulk water and hydration water dynamics, apart from a dynamical suppression that depends on the NALMA solute, exhibit the same perturbation dependence for all of the co-solvents studied. The hydrophobic solute and the high concentration water-structuring additive have the same effect on the water hydrogen bonding network. Water remains the preferential hydration of the hydrophobic side chain and backbone. We also find that the reorganization of the bulk water hydrogen bond network, upon addition of kosmotrope and chaotrope additives, is not dynamically perturbed, and that the hydrogen bond life-

time is maintained at 1 ps as in pure bulk water. On the other hand the addition of NALMA to the water/co-solvent binary system causes reorganization of the hydrogen bonds, resulting in an increased hydrogen bond lifetime. Furthermore, the solute's side chain dynamics is not affected by high concentrations of co-solvent.

2. Materials and methods

2.1. Sample preparation

Perdeuterated N-acetyl(d_3)-leucine(d_{10})-methylamide(d_3) (d-NALMA, MW 202.25) was purchased from CDN Isotopes, Canada. Normal (hydrogenated) NALMA (h-NALMA), as well as perdeuterated urea, glycerol and dimethyl sulfoxide (DMSO), were purchased from Bachem, USA. Batches of 2.5 M D-glycerol (1 mole glycerol to 22 moles H_2O), 5 M D-urea (1 mole urea to 11 moles H_2O) and 0.5 mM of $MgSO_4$ were prepared using the deuterated additive, the salt and pure H_2O. One half of each batch was used, as is, to study the influence of the additive on the *bulk water* dynamics. The other halves were used to make 1 M solutions of d-NALMA. These solutions were used to study the effects of the additives on the dynamics of the *hydration water layers*. Using a deuterated 13 M DMSO buffer (1 mole DMSO to 4 moles H_2O), perturbations of the first hydration water layer were investigated. In order to perform complementary investigations into the dynamics of the *solute* in the presence of the same co-solvents, separate buffer solutions were prepared using pure D_2O and used to dissolve the h-NALMA peptide at 1 M concentration. In order to remove small aggregates, samples were centrifuged (10 min at 10,000g) before measurement.

One molar concentration corresponds to the situation that each molecule of solute is hydrated by 55 molecules of water, so that each solute molecule is in principle associated with enough water for 1 and half to 2 hydration layers [4,20]. Hence an extended, well-structured, water shell is in place around each solute molecule, and enough molecules of water are available to be perturbed.

2.2. Experimental procedure

The quasielastic neutron scattering (QENS) experiments were carried out at the NIST Center for Neutron Research, using the disk chopper time-of-flight spectrometer DCS [32], with an incident neutron wavelength of 7.5 Å, which gives a wave vector (Q) range of 0.15 Å^{-1} < Q < 1.57 Å^{-1} and an energy resolution of 35 μeV (correlation time ≈ 60 ps) at full width half maximum (FWHM). The complementary measurements on solute dynamics were performed at the IPNS facility (Argonne National Laboratory, USA), using the quasielastic neutron spectrometer QENS [33], operating with an energy resolution of 90 μeV (correlation time ≈ 20 ps) and a Q range from 0.3 Å^{-1} to 2.6 Å^{-1}.

Samples were loaded into annular containers comprising two concentric cylinders whose radii differed by 0.15 mm for samples containing H_2O, and 0.5 mm for samples containing D_2O. Typical data collection times were of order 12 h per sample. All measurements were performed at room temperature.

All spectra were corrected for scattering by the sample container and for the contribution from the buffer when in heavy water solution. Detector efficiencies, energy resolution, and normalization were measured using a standard vanadium target. The resulting data were analyzed using the DAVE programs (http://www.ncnr.nist.gov/dave/).

3. Data analysis

The analysis of molecular dynamics by quasielastic neutron scattering is based on the very large incoherent cross section of the proton. For any sample containing hydrogen atoms, essentially all of the scattered intensity is due to them. In QENS experiments one analyses small energy changes within a window defined by the energy of the incident neutron beam and the characteristics of the spectrometer. Isotopic substitution is often used to reduce the contribution of some hydrogen atoms. For example, in parts of our study we use deuterated samples of NALMA and co-solvents, in order to reduce the otherwise dominant contribution of the hydrogen atoms of the peptide and additive when we collect the water dynamics.

A QENS spectrum generally includes contributions that are both elastic (theoretically a Dirac delta function at zero energy transfer) and "quasielastic" (i.e. inelastic, peaked at zero energy transfer). Diffusional motions contribute to the quasielastic scattering and sometimes to the elastic scattering, and the scattering associated with immobile hydrogen atoms (if any) is elastic. The spectrum is essentially the Fourier transform of the correlation function $\langle \exp[i\mathbf{Q} \cdot \mathbf{r}(0)]\exp[-i\mathbf{Q} \cdot \mathbf{r}(t)]\rangle$, where $\mathbf{r}(t)$ represents the position of an atom at time t and $\langle \cdots \rangle$ represents an ensemble average over all the atoms. The elastic component is in practice broadened by the instrumental resolution which establishes a limit to the observation of the slowest motions.

The analysis of the quasielastic component can be made within the framework of different models. At long enough times the molecular diffusion is described by Fick's law: the quasielastic line is a Lorentzian function with HWHM $\Gamma = DQ^2$, where D represents the molecular self-diffusion coefficient.

At short distances, which correspond to large values of Q, the molecule is found inside the cage formed by its neighbors. This localization in a small volume corresponds to a fixed value of Γ, from which one may extract a "residence time" $\tau_0 = 1/\Gamma$ $(Q \to \infty)$. This simplified approach to diffusion at short times, known as the jump diffusion model, does not necessarily apply but it is often a reasonable interpretation of the saturation of Γ at large values of Q.

Another model that can be considered describes the rotational motion of a hydrogen atom inside a small volume. The simplest situation corresponds to jumps between sites [34] or motion on the surface of a sphere of radius a [35]. In this particular case, the amplitude of the scattered intensity is a sum of Lorentzians weighted by spherical Bessel functions. More important and more general, the first component is elastic (because it represents the finite volume accessible to the atomic motions). The fractional intensity of this component is called the elastic incoherent structure factor (EISF). It corresponds to the form factor of the restricted volume, and it represents an exceptional case where incoherent scattering contains structural information.

In what follows, the plausibility of the application of these models to our samples is discussed in detail.

4. Results

4.1. Bulk water and hydration water dynamics

4.1.1. Translational diffusion

In order to characterize perturbations of the *bulk water* and *hydration water* dynamics due to the kosmotrope and chaotrope co-solvents, we measured the scattering from pure H_2O and fully deuterated 1 M NALMA solute in the presence of each of the following co-solvents: 5 M D-urea, 2.5 M D-glycerol, 13 M D-DMSO, and 0.5 mM $MgSO_4$. Given the small atomic fractions of solute and co-solvent molecules, and the relatively small scattering cross sections of the deuterated solute atoms as compared with that of normal hydrogen, we neglect the coherent and incoherent scattering from the solute.

Fig. 2 shows the reduced scattering function $S(Q,\omega)$, summed over all Q for bulk water and for the d-NALMA solution, with different co-solvents. For purposes of comparison the spectra are normalized to the peak maximum. As expected, the bulk water spectrum has a larger quasielastic component than the hydration water spectrum.
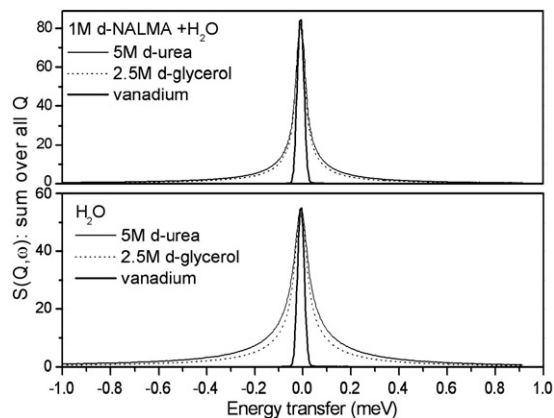

Fig. 2. The incoherent scattering function, summed over Q, for 1 M d-NALMA in H_2O (top) and in pure H_2O (bottom), together with 5 M deuterated urea and 2.5 M deuterated glycerol. The spectra are normalized to the maximum of 2.5 M D-glycerol data.

Fits to the data, as a function of Q, required a flat background and two quasielastic lines of distinctly different widths. The quasielastic scattering was well described by narrow and broad Lorentzian functions, L_1 and L_2 respectively. The narrow Lorentzian function describes the translational motion, whereas the broader Lorentzian describes movements of shorter correlation time, identified as rotational motion of water molecules [36]. These two components have been considered as independent terms, and we have assumed that the cross term, $L_1 * L_2$, is contained within the broad Lorentzian.

Fig. 3 shows the half width at half maximum associated with translation, $\Gamma_{trans}(Q)$, plotted versus Q^2 for hydration water (Fig. 3a) and bulk water (Fig. 3b), as a function of additive. Straight lines represent fits to the data, according to the simple jump model for the hydration water data, and Fick's law for bulk water data. The defined dynamics parameters have been: the residence time τ_0, which corresponds to the time necessary to break a hydrogen bond and allows jump diffusion; and the diffusion coefficient D.
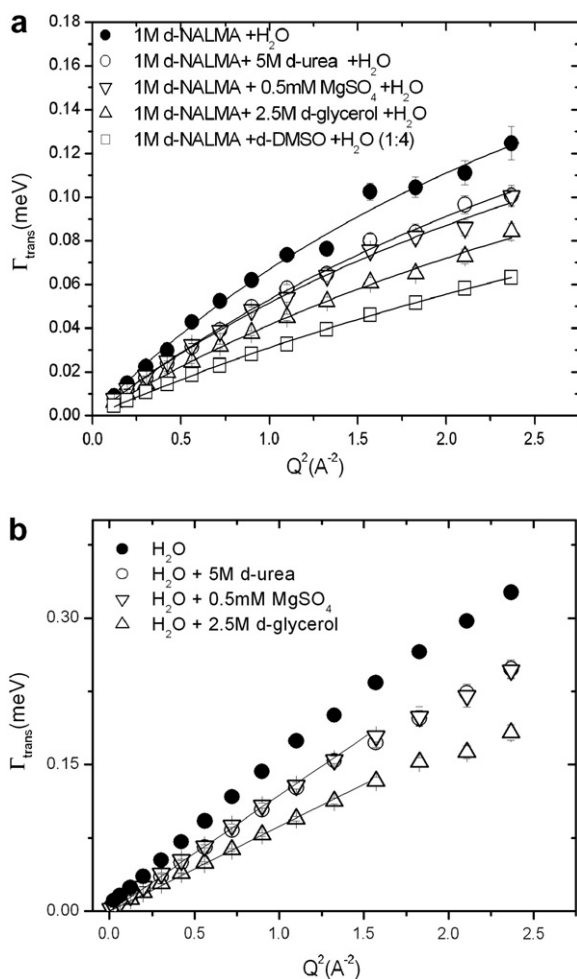

Fig. 3. Half widths at half maximum for the Lorentzian functions $\Gamma_{trans}(Q)$, plotted versus Q^2, for (a) 1 M d-NALMA and (b) bulk H_2O, together with 5 M D-urea, 0.5 mM $MgSO_4$, 2.5 M D-glycerol, and DMSO (1 mole DMSO to 4 moles H_2O). The solid lines are fits based on the random jump model in (a) and on Fick's law in (b).

Comparing the two data sets, as already observed in Fig. 2, we first of all appreciate a greater suppression of the linewidth for the d-NALMA solution than for pure H_2O. Secondly, we observe that the general perturbation of the co-solvents seems to be consistent in both systems. Glycerol, which is very soluble in water because of the OH group, induces an important perturbation on the translational dynamics component despite the relatively high concentration. We also observe that the effect on the water network perturbation of high urea concentration is equivalent to that resulting from a physiological concentration of $MgSO_4$. Finally DMSO molecules, which induce intensive water structuring with the formation of clusters of molecules, produce significant slowing down of the diffusional motion [37]. Moreover the translational dynamics of water molecules is slower in the presence of co-solvents as compared with pure water and hydration layer.

In Fig. 4 we report bulk water and hydration water translational diffusion coefficients D_{diff}, together with residence times τ_0, obtained from the fits presented in Fig. 3. For comparative purposes we also show corresponding values obtained for 1 M d-NALMA as well as the bulk water value at 298 K [36].

The diffusion coefficients shown in Fig. 4b have been inferred from the HWHMs in the low Q region of Fig. 3. The diffusion coefficients for hydration water molecules, represented by the 1 M d-NALMA solution, are considerably suppressed as compared with the corresponding bulk water coefficients, and in both cases the diffusional motion of the water is somewhat suppressed on addition of D-urea, D-glycerol, or magnesium sulfate. The important information arising from the comparison is that the dynamical parameter dependence on co-solvents shows the same behavior in both solutions. As shown in Fig. 4a, the hydration water diffusion coefficients are consistently one half of the bulk water diffusion coefficient. This behavior seems to suggest that each co-solvent structurally integrates the water network, and the introduction of NALMA molecules does not rearrange the water–co-solvent configuration. As previously observed [4,21–23], the NALMA solute strongly reorganizes the structure and dynamics of the water molecules close to its interface, and its effect on the outer layers is minor. In this particular case, the first layer reorganization is also affected by the new water/co-solvent network reorganization. Indeed our attempts to analyze the data using results for the dynamics of the first hydration shell, as described by the analysis of previous 2.0 M data [4], proved unsuccessful.

The residence time parameter τ_0, which represents the time that a proton spends at a site before jumping to a neighboring site, has only been inferred for hydration water data. For comparative purposes we show the bulk water value as obtained by Teixeira et al. [36] at 298 K.

The residence time is between 2 ps and 2.5 ps for the different solvent compositions, and the inferred value is an average of water residence times around NALMA molecules and around co-solvent molecules. The two different
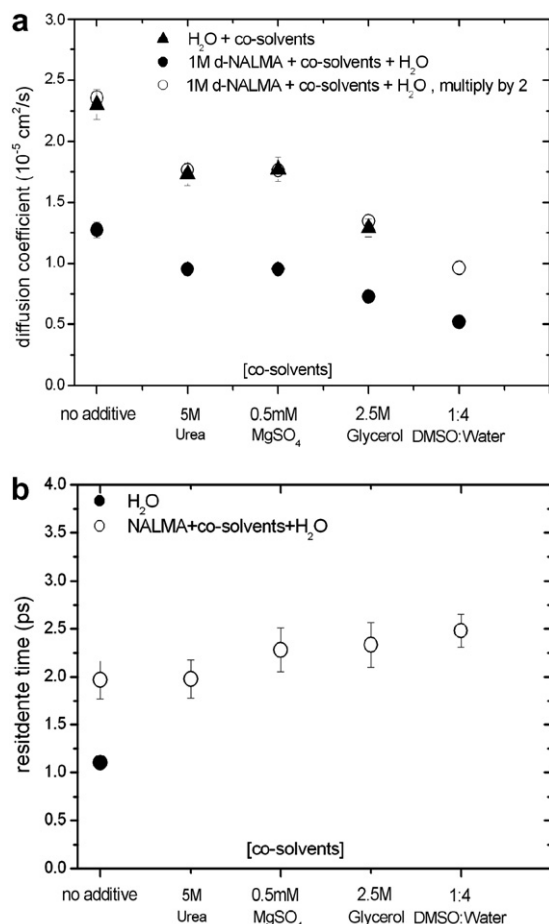
Fig. 4. Parameters inferred from the fits presented in Fig. 2. (a) Residence times inferred from the jump model for the 1 M solution as a function of co-solvent. The bulk water value has been taken from Ref. [36]. (b) Diffusion coefficients as a function of co-solvent for bulk water and hydration water (1 M d-NALMA solution). The open symbols represent the hydration water diffusion coefficients multiplied by 2.

residence times have the same time scale: indeed if we analyze the water/additive mixture using the jump diffusion model we find values of the order of picoseconds with small variations from the bulk reference value. In addition it has been shown that the residence time for water around DMSO, at the molar concentration 2 H_2O to 1 DMSO, is 4 ps at 300 K [38]. The quantity τ_0 seems to have a slight dependence (within the error bars) on the type of additive. Solutions with $MgSO_4$, glycerol and DMSO seem to have slightly longer residence times than urea and pure water, showing an ability to trap water longer. Instead the ability of urea molecules to fit into the water structure simulating two water molecules [39] may explain the equivalence to pure water.

Assuming that this weak dependence arises from water/co-solute component and taking advantage from diffusion coefficient behavior we made the hypothesis that water/NALMA residence time is constant as a function of buffer composition. Therefore the first hydration water layer only correlates with the solute. Equivalently, NALMA molecules only accept water molecules at the interface, as expected given that the used additive is a kosmotrope.

4.1.2. Rotational diffusion

In Fig. 5 we show the rotational relaxation times inferred from the rotational half-widths $\Gamma_{rot}(Q)$ as a function of co-solvent. The results have large error bars and could be better defined if an additional low resolution (short wavelength) experiment were performed to characterize a wider Q range. Nevertheless we consider that the presented data are sufficient for the general interpretation of the dynamics that we present next. Because of the short time scale we attribute the rotational relaxation time to the hydrogen bond lifetime [36].

The inferred rotational relaxation time is an average over different short time proton dynamics such as water–water, water–NALMA solute and water–co-solvent hydrogen bonds. The hydrogen bond dynamics have been characterized separately; for the pure liquid [36], for the water–NALMA solute [4], and for the water-additive binary system (this paper).

Fig. 5 shows that bulk water network hydrogen bond reorganization, upon addition of kosmotrope and chaotrope additives is not dynamically perturbed, and that the hydrogen bond lifetime is maintained at 1 ps as in pure bulk water. This evidence corresponds to the statement that these molecules integrate very well into the water network without modifying the local hydrogen bond structure. On the other hand addition of NALMA solute to the water/co-solvent binary system causes total reorganization of the hydrogen bonds, inducing a longer lifetime for hydrogen bonded water molecules. It is interesting to remark that in both binary systems (water/NALMA, water/co-solvent) the hydrogen bond lifetime is unchanged with respect to that of pure water, and it is only when we are in a more complicated configuration, such as the
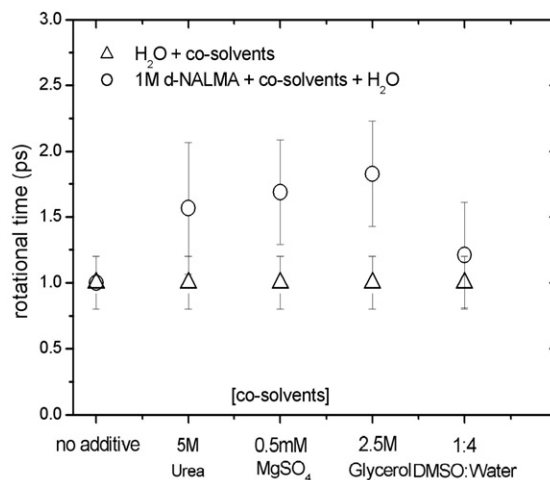

Fig. 5. Rotational relaxation times for bulk water and for 1 M NALMA as a function of co-solvent composition. The reported bulk water DMSO value is taken from Ref. [36]. Relaxation times were inferred from the broader linewidth using the Sears model approximation [31].

ternary water/NALMA/co-solvent system, that we observe significant changes. We can easily imagine that there is *interference* between the effects of hydrophobic side chains on the first hydration layer and co-solvent in the outer layer structure which leads to greater order, increasing rigidity.

From the experimental intensities, using the procedure described by Russo et al. [1], we have extracted the elastic incoherent structure factor (EISF). In this experiment, the elastic incoherent structure factor arise from the first term of the Sears model for hindered rotation [35]. Therefore the form factor is described by a Bessel function of order zero, $j_0(Qa)^2$, with values of a inferred from the quasielastic intensity fit, representing the OH distance. It is also possible to estimate from the experimental EISF the fraction of hydration atoms that are rotationally "*immobile*", on the time scale explored, and which give a constant elastic contribution, i.e. hydrogen motions slower than the experimental resolution [40].

In Fig. 6a we present the experimentally calculated EISF for the bulk water and hydration water systems as a function of additive. In Fig. 6b we show the fraction of immobile protons, as inferred from fitting the data through the relation $\text{EISF} = p + (1 - p)j_0(Qa)^2$ [40]. The average value of the radius a is roughly 1.3 Å.

In the comparison among solutes we note that they all show similar trends between bulk water and hydration water, except for the system perturbed with DMSO (Fig. 6a). A posteriori we make two interesting observations: (1) the bulk water solutions show a greater constant elastic contribution than the 1 M d-NALMA solutions; (2) in general little dependence of the EISF on co-solvent is observable, except in the DMSO case. These aspects are also apparent in the dependence of the immobile proton fraction on buffer composition and water network extension (Fig. 6b). The fact that, moving from a single hydration layer to 2–3 hydration layers (hence a more dilute system), gives rise to a change in the population of protons executing shorter rather than longer timescale reorientational motions, has already been observed and interpreted by the authors [4,22]. The behavior of the immobile protons was assigned to the activation of a new reorientational motion of water molecules that would involve exchange between *inner and outer hydration layers*.

The behavior of the EISF for DMSO in aqueous solution is markedly different compared with other hydrogen bonded mixtures. The observed deviation most likely arises from its elevated propensity to produce strong and stiff hydrogen bonds with H_2O, and from its high concentration as compared with D-urea, D-glycerol and $MgSO_4$. It is also possible that D-urea, D-glycerol, and $MgSO_4$ only affect the structure of the outer hydration layers whereas the extremely high concentration of DMSO also affects the first hydration shell, producing a more important slowing down of the related dynamics. In consequence, a higher percentage of protons are "immobilized" as compared with the other buffers.
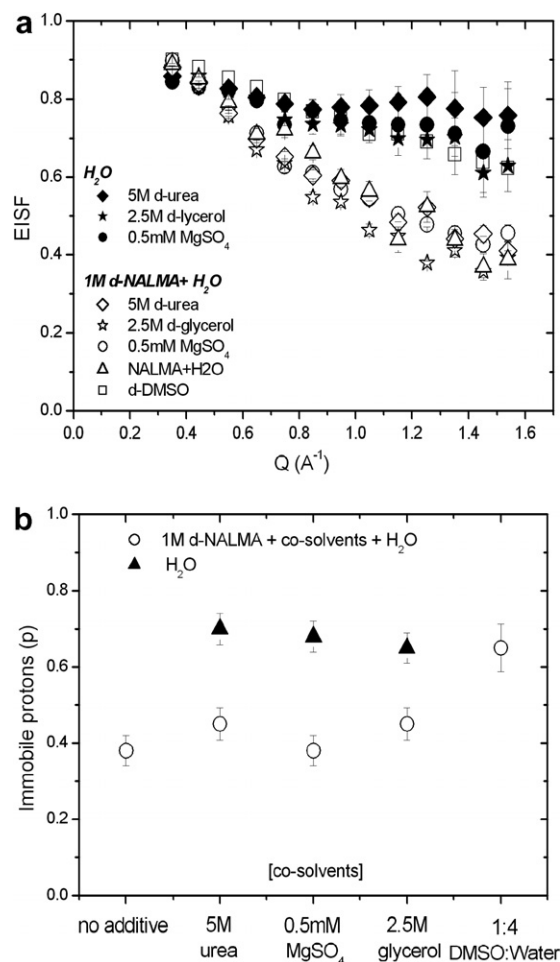

Fig. 6. (a) Elastic incoherent structure factors (EISFs) for bulk water (full symbols) and NALMA hydration water (open symbols), plotted versus Q and as a function of solute composition. The EISF corresponds to the form factor of the restricted volume explored by hydrogen atoms, and it represents the first term of the Sears model for hindered rotation [31]. (b) Immobile proton fraction as a function of co-solvent concentration for bulk water (full symbols) and hydration water (open symbols). Immobile protons are obtained by fitting the data with the model described [40].

In the limit of infinite dilution (bulk water), we also observe a high percentage of immobile protons with correlation times longer than the resolution window. In agreement with what we have discussed before, we indirectly observe the long reorientational relaxation time that was observed for water molecules in pure water, and in some cases in the binary mixtures, by dielectric relaxation experiments (15–20 ps for pure water [41], 56.8 ps for a DMSO water mixture [42]). There the measured high percentage of immobile protons in bulk water is related to the presence of an extended water network, supporting the hypothesis that there is exchange between *inner and outer hydration layers* in the case of the d-NALMA solute.

We also see that the percentage of immobile protons is largely independent of the buffer composition, suggesting that this parameter strongly correlates with the number of hydration layers: 40% for two hydration layers and an

average of 67% for *bulk water* and the DMSO buffer. In previous work we estimated that these percentages were 30% for a single hydration layer and 50% for roughly 3 hydration layers surrounding d-NALMA solute molecules [4]. Fig. 7 clearly shows that the immobile proton percentage depends linearly on the number of hydration shells. Some of the immobile protons correlate with water motions that are coupled to solute motions and in part to the additional population due to the dynamic coupling *between inner and outer hydration layers*. The coupling dynamics seems not to be affected by the variation of water structure except in the case of DMSO.

In order to explore more deeply, and to confirm some of the hypotheses made so far, we have also investigated the dynamical response of the NALMA solute under the same buffer conditions.

4.2. NALMA solute dynamics

In order to characterize the influence of co-solvents on NALMA peptide dynamics, we have measured the quasi-elastic scattering profile for 1 M h-NALMA in D_2O, in the presence of each of the following co-solvents: 5 M D-urea, 2.5 M D-glycerol and 0.5 mM $MgSO_4$.

The reduced dynamical structure factors, $S(Q, \omega)$, were analyzed by fitting the data with two Lorentzian functions, a narrow one describing the random walk diffusion of the molecules in solution, and a broad one describing the intrinsic molecule dynamics (internal dynamics). The dependence on Q^2 of the HWHM of the narrower function, $\Gamma_{diff}(Q)$, follows hydrodynamic regime behavior, yielding diffusion coefficients D_{diff} that range from 2.0×10^{-6} cm^2/s to 2.7×10^{-6} cm^2/s over the solvent composition. Fig. 8 shows the inferred dynamical parameter as a function of co-solvent composition, compared with the diffusion coefficient of 1 M h-NALMA in pure D_2O from a previous experiment [4]. As expected the diffusion coefficient is not greatly affected by the solvent composition, and the small

Fig. 8. Brownian diffusion coefficients for NALMA in D_2O, as a function of solvent composition. The diffusion coefficients were obtained from fits of the HWHM of the Lorentzian $\Gamma_{diff}(Q)$, using Fick's law.

deviation of D_{diff} for 0.5 mM $MgSO_4$, as compared with the solute in pure D_2O, can be attributed to a slightly different solute concentration.

Fig. 9 shows the dependence on Q^2 of the HWHM of the broader dynamical component, attributed to the internal dynamics. Data are only shown up to $Q = 1.7$ Å^{-1} because of the poor statistics. Despite the large error bars we can still see that the HWHM is independent of Q, and of co-solvent composition, with an average value of 0.25 meV, and that the data are consistent with the HWHM of 2 M h-NALMA in pure D_2O at room temperature (measured previously using a different spectrometer [23]). In agreement with previously published results [23], we observe localized dynamics for the internal motion. Considering the short length and time scales, this suggests rotational types of motion that probably involve the methyl groups, which represent 75% of the total protons. The side chain
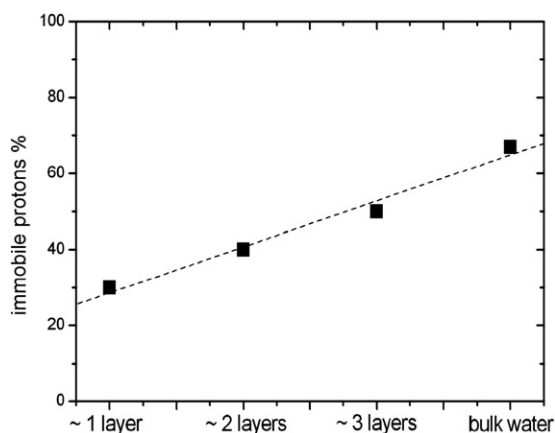

Fig. 7. The percentage of immobile protons of water molecules as a function of the number of hydration layers. The dotted line is a linear fit to the data.
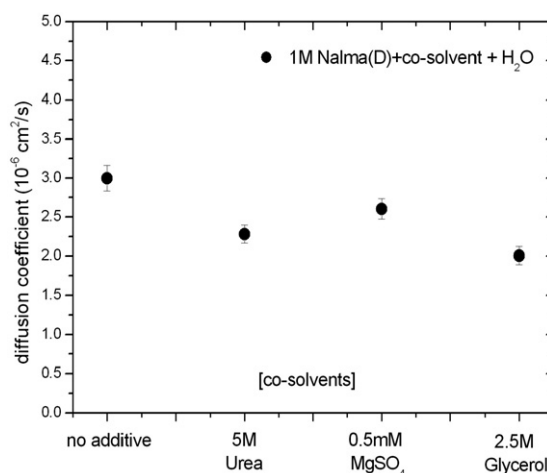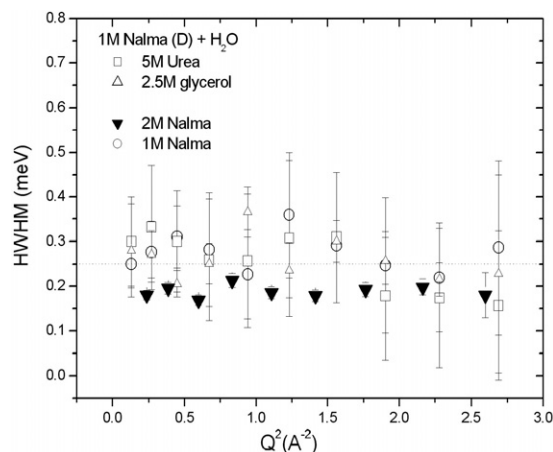

Fig. 9. HWHMs of the Lorentzian $\Gamma_{int}(Q)$ as a function of Q^2, for hydrogenated NALMA in D_2O and as a function of concentration and solvent composition. The linewidth describes the intrinsic solute dynamics, and the lack of dependence on Q suggests that the motion is confined.

and backbone motions are then dominated by methyl group reorientations. Methyl group rotation can be approximated using the 3-site jump reorientation model [30]. The corresponding residence time between methyl flips is of the order of 4 ps at room temperature, which is slightly shorter than the 2 M h-NALMA value where there is only one hydration layer. The estimated activation energy, related to the height of the potential barrier hindering rotational motion, has an average value of 7.5 kJ/mol. Activation energies depend on the type of amino acid residue, and on the residue's environment. We have observed two important facts: (1) that the internal dynamics is consistent with that of the 2 M h-NALMA solution, where only one hydration layer was present, and (2) that the internal dynamics is independent of the co-solvent in the solution. As reported in [23], the structure and the suppressed dynamics of the first and second hydration layers strongly affect the interfacing solute dynamics, constraining the explored space and suppressing translational diffusive motion. If we believe that hydration layer dynamics influences solute dynamics, we conclude that the amino acid hydration environment is not greatly altered by the presence of the additive. While similar behavior may be obvious for kosmotrope additives such as glycerol and MgSO$_4$, it is perhaps less straightforward for urea when used at such concentrations. Nevertheless, urea has been seen to interact directly with aliphatic and aromatic side chains [43,44] and is thought to assist hydration of hydrophobic residues, increasing their solubility. This would happen at high concentration (at least 1 mole urea to 4 moles H$_2$O). such that urea is readily available for the hydration shell [39]. Considering the water dynamics results and the relative high D-urea concentration (1 mole urea to 11 moles H$_2$O), we assume that urea is only present in the second hydration layer and behaving as a kosmotropic co-solvent.

5. Discussion and conclusions

Chaotropic molecules are often used to unfold proteins and destabilized hydrophobic aggregates in aqueous solution, increasing hydrophobic particle solubility [45]. Kosmotropes are protein stabilizing solutes which decrease the solubility of hydrophobic particles and stabilize their aggregates.

In this study we investigated the impact, on aqueous solutions of high concentration hydrophobic bio-molecules, of kosmotrope (ionic and non-ionic) and chaotrope molecules. The objective was to understand how the short time dynamical properties of bulk and hydration water can be affected on introduction of chaotrope and kosmotrope solutes in the solution, and how dynamical properties of highly soluble hydrophobic peptides can be indirectly modified. We were also interested to investigate the role of hydrogen bonds in water and to what extent it is modified by the addition of hydrogen bonded molecules. In order to achieve our goal we worked at a solute concentration such

that we could investigate the very first hydration layer and avoid the solubility problem when adding kosmotrope. We used ionic and non-ionic kosmotropes spanning a wide range of concentrations and never we observed a decrease in the solubility of a 1 M NALMA solution. Hydrophobic NALMA peptides are soluble in aqueous solution up to 2 M, and it is plausible that we would only have observed a collapse of the system at this high concentration.

Using different approaches to the problem we have found that the dependence of the translational dynamical parameter on co-solvents shows consistent behavior in bulk and hydration water. Each co-solvent structurally integrates the water network, and the introduction of NALMA molecules does not rearrange the water–co-solvent configuration, but only strongly reorganizes the structure and dynamics of the "free" water molecules close to its interface. Hydration water diffusion coefficients are consistently one half of the bulk water diffusion coefficient and water/NALMA residence times can be considered constant as a function of buffer composition. Peptide side chain and backbone motions, which are dominated by methyl group rotations, also seem to be completely independent of co-solvent in solution. We conclude that solute and additive produce completely independent perturbations of the water network, that the environment at the interface of a NALMA molecule is not greatly perturbed by co-solvents, and that no molecules other than water are found at its surface. Preferential hydration of surfaces is a consequence of the behavior of kosmotropes in solution, and in the present work chaotropic urea molecules act kosmotropically because of the relatively high urea concentration, and its ability to easily integrate the water network and the high solubility properties of NALMA. The DMSO additive might be an exception given its high concentration.

How the dynamical properties of water are affected and slowed down by both glycerol and dimethyl sulfoxide is consistent with previously published work on the dynamical properties of water/DMSO mixtures at high concentration [38,42,46,47] and the properties of water/glycerol mixtures [48–50]. Relatively small, non-ionic kosmotropes have both hydrophobic and hydrophilic sides which allow them to fit into the water network. The hydration shells of these molecules are quite similar and they present low density cages of water around methyl groups (DMSO) and CH groups (glycerol), surrounded by strong hydrogen bonds, either to the oxygen (1 hydrogen bond possible for a single DMSO molecule, acceptor) or to OH groups (3 hydrogen bonds possible for a single glycerol molecule, donor). However water–DMSO hydrogen bonds tend to be stiffer and stronger, as compared with water–water hydrogen bonds [46], consistent with the strong suppression of the water dynamics. A comparison of the dynamical results for water/glycerol and water/DMSO demonstrates that solvent viscosity is a very important parameter that controls water and the suppression of protein dynamics [51,52].

We also observed that the impact on the water dynamics of an ionic kosmotrope is stronger that of non-ionic kosmotrope additives. Ionic kosmotropes have properties that differ from those of non-ionic kosmotropes; they interact strongly with water molecules and are polarize their local environment, increasing the structure in their first hydration shell and promoting the formation of denser water. The low physiological concentration of 0.5 mM $MgSO_4$ produces a suppression of the diffusion coefficient equivalent to a decrease in temperature of 11 K with respect to a pure 1 M solution, and 15 K with respect to bulk water [53]. The residence time value corresponds to a decrease in temperature of 22 K with respect to the pure 1 M solution, demonstrating the ability of ionic kosmotropes to polarize and eventually trap water in a puckered arrangement [31]. In addition, wide angle diffraction data on a 1 M NALMA solution shows an enhancement in the intensity of the characteristic correlation peak at 0.8 $Å^{-1}$ when 0.5 mM $MgSO_4$ was added to the solution but not when non-ionic kosmotropes were added [54]. Salts such as $MgSO_4$ can influence physicochemical properties and interactions between proteins due to the ionic strength effect, and at high concentration they can drastically alter the structure of water and in consequence the hydrophobic interaction and protein stability.

As a matter of interest we also report that a 5 M solution of the chaotrope D-urea has, both for hydration water and for bulk water, very similar translational dynamics to that of the ionic kosmotrope solutions, slightly diverging only in the value of the residence time. The urea residence time is shorter and very similar to that of pure liquid water, eventually justified by the lack of electrostatic interaction or simply a local structure modification. Despite the doubled water/co-solute molar ratio as compared with D-glycerol, the urea/water dynamics is less suppressed due to lower viscosity and effortless integration into the water network. Urea incorporates into water forming strong hydrogen bonds with water at both the amine and carbonyl head groups (a total of six hydrogen bonds for a single urea molecule, two donors and four acceptors). Indeed neutron diffraction experiments on high concentration urea/water mixtures show, through the pair correlation function, that the tetrahedral water structure is not affected [39]. The same structural study advances the hypothesis that urea substitutes for water molecules in the water hydrogen bond displacing two water molecules, keeping a similar number of hydrogen bonds relative to water, and providing a similar range of geometries for the collection of hydrogen bonds [39]. Regardless of the easy integration of the urea molecules in the bulk and hydration shells network, something changes in the overall configuration since we have observed stiffening in the translational dynamics. We can possibly interpret the results as a modification of the translational cage around the water molecules, becoming smaller and more rigid due to an intensification of the interactions between water molecules and their surrounding as suggested by the results of molecular dynamics sim-

ulations by Idrissi et al. [55]. These workers do not find any significant dynamical difference from bulk water at our working concentration, which may be because we used deuterated urea. Deuterium is heavier than hydrogen and can create stronger and more stable hydrogen bonds. Certainly the stability of proteins in D_2O solution is greater [56,57] and the unfolding dynamics is shifted to higher temperatures or co-solvent concentrations than in the case of light water [58,59].

The NALMA hydration environment and side chain motions do not seem to be more perturbed by urea than in the presence of other additives. Urea acts as a denaturant supporting hydration of hydrophobic residues, which would happen only at very high concentrations when urea is ready to be available for hydrophobic interaction [39]. The NALMA hydrophobic peptide is highly soluble in water. The hydrophobic side chains are completely exposed to water, and supporting hydration from urea is unnecessary. At a ratio of 1 mole urea to 11 moles H_2O it is likely that urea is not close to the interface with the hydrophobic residue even if it is incorporated into the solvation shells.

We affirm that the nature of a solute, whether kosmotrope or chaotrope, strictly depends on the physical properties of the solution.

Analysis of the rotational relaxation time also shows interesting features. The water rotational relaxation time remains unchanged with respect to bulk water in the binary system, and is slowed down in the more complex NALMA/water/co-solvent mixtures. The first piece of evidence seems to indicate that the water structure in the mixture mostly retains the properties of the bulk. On the other hand, the second piece of evidence reinforces the fact that the water/co-solvent mixture somehow has some local effect on hydrogen bonds. This would seem to suggest that the measured average value, in bulk water, arises from dominant contributions from free water molecules in tetrahedral configurations. The water/DMSO fast rotational time, at this high concentration, has been interpreted by Cabral and coworkers [38] who also simply proposed that the value is an average over two different proton dynamics: water–water and water–DMSO. They then suggested that the correspondence between the proton-hindered rotational relaxation time and the hydrogen bond lifetime was no longer legitimate, given also that molecular dynamics calculations of hydrogen bond lifetimes for water–water and water–DMSO hydrogen bonds produced longer times, 5 ps and 10 ps respectively.

We have also observed that a long reorientational relaxation occurs, and that the fraction of protons involved correlates linearly with the number of hydration layers. The percentage of protons involved in this motion reach its maximum in the bulk water solutions. Extending the water network exchange of H_2O molecules among different hydration layers is allowed, and occurs not only through translation but also through reorientational displacements. Certainly some of this slow water corresponds to molecules that are coupled to solute motions and/or to water that is

strongly trapped in the first hydration shell. Rezus and Bakker [60] show, through polarization-resolved mid-infrared pump-probe spectroscopy, that even at high urea concentrations, such as 8 M, the reorientational dynamics of most water molecules is the same as in pure liquid water, so that urea has a negligible effect on hydrogen bond dynamics and vibrational relaxation of water molecules. Together with the fast orientational dynamics they also show the presence of a slow dynamics, which becomes more important at increasing urea concentrations. Given the linear urea concentration and the dynamical probe, they attribute this component to the bound water in the urea hydration shell.

Our results also confirm that a hydrophobic side chain requires an extended hydration layer of solvent in order to attain the liquid-like dynamical regime [23]. Indeed when side chains are hydrated by one or two hydration water layers the only allowed motions are confined, and often attributed to simple rotations of methyl groups. The structure and the highly suppressed dynamics of the first hydration layers [4] strongly affect the interfacing solute dynamics, constraining the explored space and (of most importance) suppressing translational diffusive motion. We have shown in previous publications that even when roughly two hydration layers are in place the observed dynamical suppression of hydration water arises from the strong contribution of the first layer. Then a more relaxed water network, which can be attained with several hydration shells, is necessary to allow the side chain to exhibit the full range of motions.

Acknowledgements

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